

CHANGES IN *STREPTOMYCES HYGROSCOPICUS* 155 ENDOPEPTIDASE AND AMINOPEPTIDASE ACTIVITY AND HEAT RESISTANCE UNDER STARVATION AND INCREASED TEMPERATURE

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Summary

The influence of temperature stress and starvation for amino acids, glucose and phosphates, on the heat resistance of mycelium and endo- and amino-peptidase activity of Streptomyces hygrosopicus 155, was studied. The strongest growth inhibition was determined at temperature elevation from 30° to 39° C and at starvation for amino acids. Also these stress treatments mostly induce the heat resistance of the mycelium. A correlation between the intracellular endo- and aminopeptidase activity and decrease in biomass yield was registered. The process of catabolization of proteins, during the adaptation to stress treatments, depends on energy and is stimulated by the presence of Mg²⁺ ions.

Introduction

Streptomyces are microorganisms undergoing a complicated life cycle: formation of substrate mycelium, air mycelium and spores. Streptomyces spores are forms of propagating and surviving unfavourable life conditions and they express considerably higher heat resistance as compared with the mycelium structures [5]. Sporulation is a long process and in most cases lasts several days. Living cells, however, possess the ability to perform fast molecular response as a result of the action of sublethal temperatures or starvation. The adaptation to these leads to increase heat resistance [17].

The sequence of the intracellular alterations coming in the microorganisms under the action of different stress factors has not profoundly been studied yet. But in the cases of starvation and temperature shocks biosynthesis of specific adaptation proteins is well known for several species [11,12]. In order a biosynthesis of these latter proteins to be performed, it is necessary for the intracellular

pool of amino acids to be filled. This is realized through catabolization of the abnormal proteins or of those not participating in the cell metabolism.

Among the studied intracellular proteolytic enzymes in *Escherichia coli*, two were found to influence the adaptation to heat stress: La-protease and ClpP proteinase [4, 8]. Both enzymes are ATP dependent and the second catabolize 1 molecule ATP through hydrolysis of 1 peptide bond [8].

Homologous to Clp proteases exhibiting high degree of similarity in size as well as in immunological relations to antiClpP antibodies have been found in a number of prokaryotes and eucaryotes [2]. G7 found in *Bacillus subtilis* is a not inducible general stress protein similar to ClpP of *E. coli*. Its temperature stress as well as in case of other drastic changes in environment [14].

The role of the intracellular proteinases in the adaptation processes of streptomyces to nutritional shift down or temperature stress

has scarcely been studied at all. There are few data connected with the antibiotic biosynthesis or sporulation that enlightened indirectly this question [10,13].

The present work is a preliminary study

on the changes taking place in the intracellular proteinase activity and induction of heat resistance of mycelium under the action of temperature shocks and peptone, phosphate and glucose starvation in *S. hygroscopicus* 155.

Materials and Methods

Strains. The present studies were performed with streptomyces strain *S. hygroscopicus* 155 (NBIMCC 227).

Media. Medium Gause I was used for sporulation of the studied strain. Cultivation was performed in rich medium (FM) which provided high growth rate and consisted of (g/l): NH_4Cl - 1, $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ - 0.5, NaCl - 0.5, peptone - 10, glucose - 20, supplemented with 100 ml 500mM potassium-sodium phosphate buffer, pH 7.2 and 1ml solution of micro-elements. The microelements solution was composed of (for 100 ml) of MnCl_2 - 0.1 g, ZnSO_4 - 0.1 g, FeSO_4 - 0.1 g.

Also, modified variants of FM medium for performance of different nutritional starvations were used. The media in which the starvation experiments were performed had the same composition as FM without peptone (FM-P), without glucose (FM-GI) or without phosphate buffer (FM-Ph). During phosphorous starvation, the medium was buffered with 50mM TRIS-HCl buffer, pH 7.2.

Obtaining of spore material. Fourteen-day-old spores obtained in Gause I medium at 30°C were suspended in physiological solution and filtered through cotton to obtain monospore suspension. The suspension was washed twice with physiological solution, the spores in concentration 10^9 /ml were suspended in 20% (v/v) solution of glycerol and kept at -20°C.

Cultivation. Two-stage cultivation was performed. 500 ml flasks containing 99 ml FM medium were inoculated with 1ml spore suspension in concentration 10^9 /ml and cultivated for 24h. The obtained culture was used as inoculum. 95ml FM medium kept at 30°C in order to avoid induction to adaptive alterations due to temperature changes, were inoculated with 5 ml inoculum. In order to obtain the aeration needed 500ml flasks with 100ml liquid medium were used. Cultivation was per-

formed on rotary shaker (240rpm) at 30°C excluding the experiments for the heat stress.

Stress treatment. Forty-eight-hour-old culture was subjected to the following stress conditions. Heat shock was performed through increase of the cultivation temperature from 30° to 39°C (for sublethal stress). Starvation for substrates was realized through sterile filtration of the mycelium from one flask, washing with 50ml of the appropriate starvation medium and transferring in 500ml flask containing 100ml of the same medium, kept at 30°C.

Dry weight determination. The quantity of the obtained biomass was determined according to Ochi [11].

Viability evaluation. At appropriate hours, 1 ml aliquots were withdrawn and heated at 52°C for 30min. After suitable dilution in physiological solution, 0.1ml of the samples was inoculated in Gause I medium. The formed colonies were recorded on the 4th day. The viability was calculated as a percentage of the number of the colonies formed from the not treated at 52°C sample. The number of colonies of unheated sample was accepted as 100%.

Cell-free extract (cytosole fraction) preparation. The stressed culture was filtered through paper filter, washed with 20 ml 5M KCl, washed twice with distilled water, each time with 20 ml and finally washed with 100 ml physiological solution. The obtained mycelium for preparation of cytosole fraction was kept at - 20°C. The cell-free extract was obtained by grinding with quartz sand for 30 min. The sample was centrifuged at 5000 x g for 5 min in order to pellet the disrupted mycelium particles. The membrane fraction was sedimented by centrifugation at 15 000xg for 40 min. The obtained samples were stored at - 20°C. All procedures for obtaining of cytosole fraction were performed at 4°C.

Protein content determination. The protein content was determined according to the method of Lowry et al. [7].

Enzyme activities assay. The total intracellular endopeptidase (EP) activity was determined according to El Soda et al. [6]. The reaction mixture contained 2.5 ml 50 mM potassium phosphate buffer pH 7.0, 0.1 ml substrate (5 mg succinyl-L-Phe-pNA in 1 ml methanol) and 0.4 ml cell-free extract. One unit of activity is equal to the amount of the enzyme that releases 1 μ M *p*-Nanilide per hour at 30°C.

The aminopeptidase (AP) activity was determined according to El Soda et al. [6]. The reaction mixture consisted of 2.5 ml 50 mM potassium phosphate buffer pH 7.0, 0.1 ml substrate (6.4 mg L-Leu-pNA in 1 ml methanol) and 0.4 ml cell-free extract. The amount of enzyme that releases 1 μ M *p*-Nanilide per minute at 30°C is equal to one unit.

Results reproducibility. Each presented result is an average value from two separate experiments.

Results and Discussion

1. *S. hygroscopicus* 155 growth under peptone, phosphate and glucose starvation and sublethal temperature

In order to study the influence of starvation for different nutrients (glucose, phosphates and peptone) and sublethal temperature (39°C) on *S. hygroscopicus* 155 growth, FM medium providing long growth phase was used.

The starvation for glucose, phosphate and organic nitrogen as well as the applied heat stress expressed an inhibitory effect on the growth in different extent. The obtained results are presented in Fig. 1a. The strongest effect on growth was determined at temperature elevation from 30° to 39°C. Temperature of 39°C was sublethal for *S. hygroscopicus* 155, because its growth continued 72 h after

the temperature shift up. From the 3 kinds of starvation, the transfer of the culture on peptone deficient medium mostly inhibited the growth and the biomass yield was the lowest. Starvation for phosphate had weaker effect and the growth phase was the longest (up to 120 h). A similar model of influence of starvation for inorganic phosphate on growth was also observed in marine *Vibrio* strains [9] and *Streptomyces griseus* [1]. No stationary phase of the strain was observed under all the tested cultivation conditions and lysis of the cell took place immediately after the end of the growth phase. This fact was probably due to the biological peculiarities of the species, because it was observed both during notlimited and limited growth.

2. Heat resistance of the *S. hygroscopicus* 155 mycelium

Streptomyces possess high heat resistance of the submerged and surfaced spores unlike of the mycelium [5]. of *S. hygroscopicus* 155 failed to form submerged spores under three studied starvation condition. At the chosen sublethal temperature challenge (30 min at 52°C), twentyfour-hour-old mycelium of *S. hygroscopicus* 155 cultivated in FM expressed viability of 16.1% \pm 1.4%. The thermostability had not varied considerably for 8 hours and was used as a control in the heat resistance induction experiments (Table 1.). *S. hygroscopicus* 155 had rather high temperature resistance of mycelium. This fact is possible to due to the protective effect of glucose and peptones in FM medium.

During substrate limitation of growth the

heat resistance *S. hygroscopicus* 155 showed 2 picks, while at temperature elevation to 39°C only one was observed (Table 1.). The induction of higher thermostability of *S. hygroscopicus* 155 mycelium by the tested stress treatments was expressed as a percentage of strain subjected to definite stress factor viability (30 min at 52°C) against its viability (30 min at 52°C) when cultivated in FM medium at 30°C. From all tested nutritional down shifts (Fig. 1b) only starvation for phosphate did not lead to increase of the heat resistance. An induction of higher thermostability was observed in rest of the cases. The highest increase was found when the strain was cultivated at 39°C and subjected to starvation for organic nitrogen. The induction of higher heat resistance in *S. hygroscopicus*

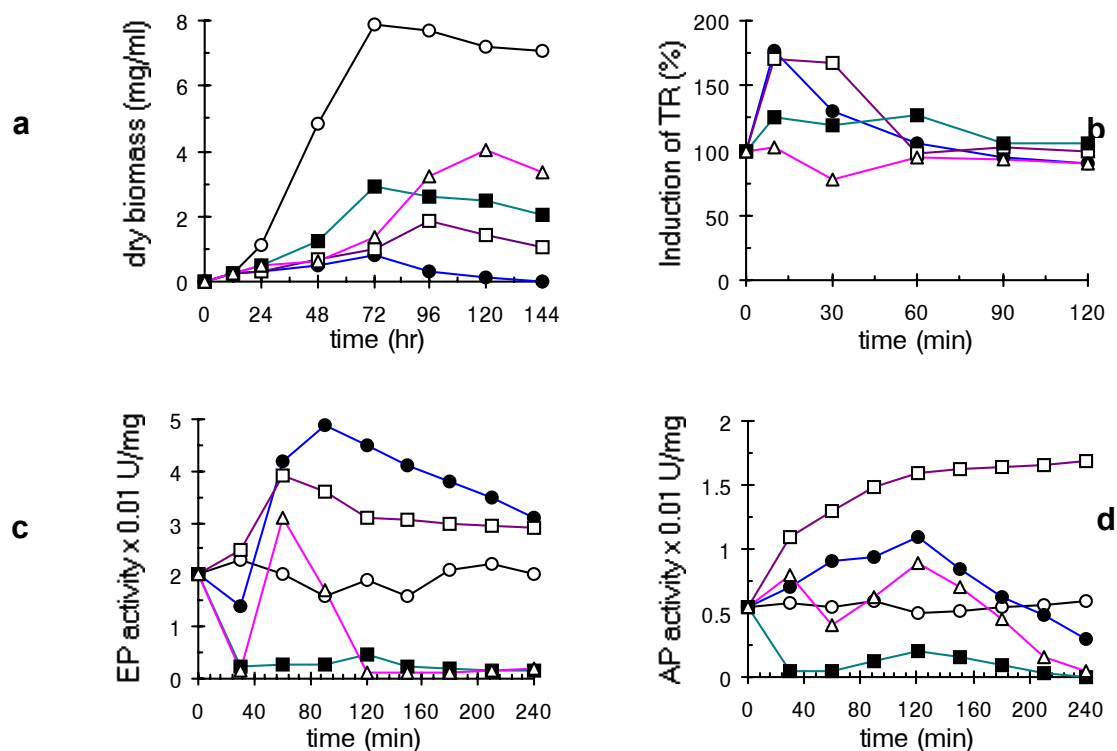


Fig.1. Influence of different types of substrate starvation and temperature stress on: the growth (a); the induction of termoresistance (TR) (b); the endopeptidase (c) and aminopeptidase (d) activity of *S. hygroscopicus* 155. Growth in: FM, 30°C (—○—); FM, 39°C (—●—); FM-P, 30°C (—□—); FM-G, 30°C (—■—) and FM-Ph, 30°C (—+—). The induction of termoresistance is expressed as a percentage of stressed mycelium viability (at 52°C for 30 min) against the untreated culture viability (at 52°C for 30 min).

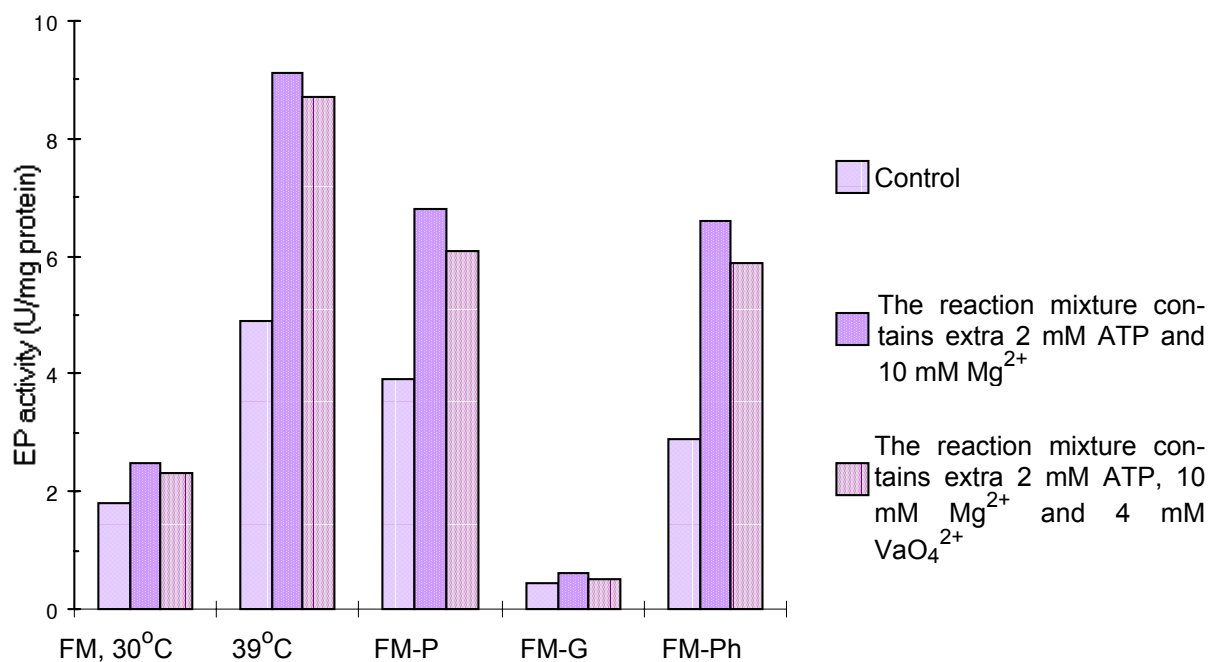


Fig. 2. Influence of ATP, Mg²⁺ and VaO₄²⁺ on the endopeptidase activity in a cell-free extract of a twenty four-hour-culture of *S. hygroscopicus* 155 at 30°C. The culture is subjected to: FM-P for 60 min; FM-G for 30 min; FM-Ph for 60 min and 39°C for 60 min.

155 was of transitional nature. This induction reached its maximal values at 39°C on the 10 min after the temperature alteration. Similar was the model of induced thermostability in starvation for organic nitrogen, but the higher values had been kept for 30 min. A delay in

heat resistance stimulation was observed in the culture starving for glucose. The highest values were found at the 60 min. In all 4 stress factor treatments the survival at lethal temperatures reached its initial values or lower 4h after alteration of the cultivation conditions.

Table 1. Viability of 24h mycelium of *S. hygrosopicus* 155C (at 52 °C for 30 min) cultivated in FM medium or in peptone deficient medium (FM-P), glucose deficient medium (FM-GI) and phosphate deficient medium (FM-Ph) at 30°C and in FM medium at 39°C

Post-treatment period (min)	Viability (%)				
	FM, 30°C	FM, 39°C	FM-P, 30°	FM-GI, 30°	FM-Ph, 30°
0	15.6	14.9	16.7	15.1	17.2
10	17.2	27.8	29.6	17.5	18.5
30	17.4	20.3	30.8	16.33	12.2
60	16.3	15.9	13.3	20.8	16.8
90	15.2	15.1	14.2	14.6	15.3
120	15.0	14.6	15.8	12.2	14.9
150	16.9	13.7	15.5	12	14.2
180	17.5	13.2	13.4	11.7	14
210	15.0	13.9	12.1	10.4	13.1
240	15.2	14.1	11.9	10.1	12.7

3. Changes in the intracellular endo- and aminopeptidase activities

The studied stress treatments expressed different effect on *S. hygrosopicus* 155 intracellular proteins catabolization. The results concerning the changes in the intracellular endopeptidase and aminopeptidase activities during the initial adaptation stages are presented in Fig. 1c and 1d. The transfer of the twentyfour-hour-old culture of *S. hygrosopicus* 155 from rich medium (FM) into peptone deficient medium led to increase of the intracellular proteinase and aminopeptidase activities immediately after the beginning of the experiment. The activation of the intracellular proteinases under conditions of starvation for amino acids was connected mainly with the need for the intracellular amino acid pool to be filled for biosynthesis of new specific adaptation proteins. When increasing the cultivation temperature or starving for phos-

phorous the rise of the proteinase activity took place after the initial decline (at the 30 min). The highest values of the proteinase activity were observed by the increase of the strain cultivation temperature and in peptone deficient medium. Starvation for organic nitrogen, however, has expressed a stronger effect on the aminopeptidase activity than the heat stress. In case of deficiency in the cell of one or more amino acids, the uncharged tRNA is attached to ribosome during protein synthesis and the peptide elongation is interrupted. As a result a great number of low molecular weight peptides which are toxic for the cell are accumulated in mycelium. Degradation of these peptides is performed by aminopeptidases, which fact could explain the high aminopeptidase activity of the strain when cultivated in peptone deficient medium.

When transferred in glucose deficient medium *S. hygrosopicus* 155 exhibited strong decrease of its proteinase as well as aminopeptidase activities. Also, in number of microorganisms, ATP dependent proteinases took place in the catabolization of the intracellular proteins. Due to this fact probably, the cell starvation for energy is a reason for the low endopeptidase activity and the low aminopeptidase activity is a consequence of it.

In order to check the role of the energetic provision of the cell for the proteinase activity the effect of ATP on the total intracellular endopeptidase activity was studied. The analysis of the obtained results (Fig.2) showed that the protein catabolization was dependent on ATP concentration and presence of Mg^{2+} . The endopeptidase activity was increased about 2 times when they were added to the reaction mixture. The vanadate ions inhibited the ATP-ases' ability to hydrolyze ATP. In our experiments they expressed low effect on the proteinase activity in the presence of ATP which fact showed that the

main proteinase activity in heat stress and substrate limitation of growth (excluding glucose one) was activated by ATP probably due to allosteric alterations, not to consumption of energy released by ATP catabolism. Some minor endopeptidases are likely to act as energy dependent, thus bringing to a slight decrease of the proteinase activity in the presence of vanadate ions.

A relation between induction of heat resistance and changes in endo- and aminopeptidase activity of *S. hygrosopicus* 155 had not been established by the performed experiments. The increase of the activity of these enzymes especially of the aminopeptidases during initial adaptation correlated with the decrease in biomass yield, observed during starvation and heat stress. Multilocus analysis of the intracellular proteinases and aminopeptidases will provide with more extensive information concerning the number and kind of enzymes catabolizing proteins which act during adaptation.

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